Kidney International, Vol. 68 (2005), pp. 2462–2472

GENETIC DISORDERS – DEVELOPMENT

Interaction between *Rf-1* and *Rf-4* quantitative trait loci increases susceptibility to renal damage in double congenic rats

SABINE J. VAN DIJK, PATRICIA A.C. SPECHT, MICHELLE M. LUTZ, JOZEF LAZAR, HOWARD J. JACOB, and ABRAHAM P. PROVOOST

Department of Paediatric Surgery, Erasmus MC, Rotterdam, The Netherlands; and Department of Physiology, Department of Dermatology, Department of Paediatrics, and Human and Molecular Genetics Centre, Medical College of Wisconsin, Milwaukee, Wisconsin

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Background. Five quantitative trait loci (QTLs), *Rf-1* to *Rf-5*, were found in Fawn-Hooded hypertensive (FHH) rats influencing susceptibility to renal damage. Previously, we found that single transfer of the *Rf-1* QTL from FHH rats onto the renal-resistant August × Copenhagen Irish (ACI) strain caused a small increase in renal susceptibility. To investigate the separate role of the *Rf-4* QTL and its interaction with *Rf-1*, we generated a single congenic strain carrying *Rf-4* and a double congenic carrying both *Rf-1* and *Rf-4*.

Methods. Differences in renal susceptibility between ACI, *Rf-1A*, and *Rf-4* single congenics and *Rf-1A+4* double congenics were assessed using four different treatments: control (two-kidney), two-kidney with L-arginine analogue N-nitro-L-arginine methyl ester (L-NAME)–induced hypertension, unilateral nephrectomy, and unilateral nephrectomy + L-NAME. In separate experiments, renal blood flow (RBF) autoregulation was compared between two-kidney ACI and congenic rats.

Results. Compared to ACI, *Rf-1A* rats developed more renal damage, while *Rf-4* rats did not. The most severe renal damage was found in the *Rf-1A+4* double congenic rats. Analysis of variance (ANOVA) demonstrated a significant interaction between the *Rf-1A* and *Rf-4* QTLs. The magnitude of the interaction varied with the type and duration of the treatment. The RBF autoregulation was impaired in *Rf-1A* single and *Rf-1A+4* double congenics, while in *Rf-4* single congenics it was similar to that of ACI controls.

Conclusion. These findings indicate that the *Rf-1* QTL directly influences renal susceptibility and autoregulation. In contrast, the *Rf-4* QTL shows no direct effects, but significantly increases susceptibility to renal damage via an interaction with *Rf-1*.

Chronic kidney disease is assumed to be a complex polygenic disease [1–5]. Chromosomal loci, as well as specific genes, have been identified in various inherited forms of renal disease [6, 7]. However, finding the genes involved in the more complex forms of human end-stage renal failure (ESRF) has been more arduous. Linkage analysis has identified some chromosomal regions possibly involved in diabetic and nondiabetic forms of nephropathy, while candidate gene analyses have tested several genes with limited success [4, 8, 9]. Studies in inbred rat strains might help to decrease the number of candidate genes, which could facilitate finding genes in human studies.

Inbred rat strains also vary widely in their susceptibility to develop renal damage. Our studies involve the Fawn-Hooded hypertensive (FHH) rat, prone to develop mild systolic hypertension and marked proteinuria (UPV), albuminuria (UAV), and focal and segmental glomerulosclerosis (FSGS) at relatively young age. Male FHH rats die of ESRF within a year and a half if not treated. [10, 11] The FHH strain is well characterized by numerous physiologic and histologic studies [12–19]. Crosses between FHH and the proteinuria-resistant August × Copehnagen Irish (ACI) rat revealed the presence of five quantitative trait loci (QTLs) linked to UPV and other parameters of renal damage. These QTLs were named *Renal failure-1* (*Rf-1*) to *Rf-5* [20, 21]. It is surmised that each of these QTLs contains gene(s) that play a role in the development of progressive renal damage in the FHH rat.

Even though the nature of the genes have not yet been identified, the separate role of each QTL in the initiation and progression of renal damage can be studied in congenic rat strains that have a *Rf* region of the FHH rat introgressed into the genomic background of the proteinuria-resistant ACI rat. Next, interactions between the QTLs can be studied in double and multiple congenic strains. Previously, we reported about the susceptibility to renal damage in ACI.FHH-*Rf-1B* (*Rf-1B*) and ACI.FHH-*Rf-5* (*Rf-5*) single congenic rats [22, 23]. Since the ACI rat is resistant to renal damage even when made hypertensive, we need to stress the kidney to initiate the

Key words: end-stage renal failure, L-NAME, congenic rats, gene-gene interaction, albuminuria.

Received February 10, 2005
and in revised form April 12, 2005, and June 8, 2005
Accepted for publication July 7, 2005

renal failure phenotype and be able to study the effect of a single QTL in the ACI background. The *Rf-1B* congenic rats developed significantly more UPV and UAV than the ACI progenitor strain. This difference was most pronounced following unilateral nephrectomy combined with L-arginine analogue N-nitro-L-arginine methyl ester (L-NAME)–induced hypertension.

In *Rf-1B* congenic rats, renal autoregulation was impaired to the same extent as the parental FHH rat [18]. In the FHH rat, the impaired renal autoregulation, resulting in an elevated intraglomerular pressure (P_{GC}) is thought to be an important mechanism in the early development of renal damage [13, 15, 19]. In contrast to the *Rf-1B* congenic rats, the *Rf-5* single congenic strain showed no increase in renal susceptibility and a normal renal blood flow (RBF) autoregulation [23]. In the *Rf-1B* congenic, the levels of UPV and UAV following unilateral nephrectomy and L-NAME treatment were much less than those found in FHH [14]. This indicates that the *Rf-1* QTL only accounts for part of the renal damage in FHH rats. Our previous linkage analysis suggested complex interactions between the QTLs. With the exception of *Rf-1*, the other *Rf*-QTLs by themselves showed little effect on UPV. However, a marked increase in UPV level was noted when these QTLs were combined with *Rf-1* [21]. Direct evidence for such an interaction can be obtained by studying double congenic rats. Interactions between blood pressure QTLs in rats have been described previously [24, 25].

In the present experiments we wanted to test the presence of an interaction between the *Rf-1* and *Rf-4* QTLs, as predicted from the linkage analysis [21]. Therefore, we compared the renal susceptibility between the ACI progenitor strain and three congenic strains (i.e., ACI.FHH-*Rf1A,* ACI.FHH-*Rf-4* single congenics, and ACI.FHH-*Rf1+4* double congenics). We tested the hypothesis that a gene-gene interaction occurs between the *Rf-1* and *Rf-4* QTLs increasing the susceptibility to renal damage.

METHODS

Congenic and control rat strains

For the experiments, single congenics ACI.FHH-(*D1Rat74-D1Rat90*) (*Rf-1A*), ACI.FHH-(*D14Mit11-D14Rat82*) (*Rf-4*), double congenic ACI.FHH-(*D1Mit18-D1Rat90*)/(*D14Mit11-D14Rat33/D14Rat65-D14Rat90*) (*Rf-1A+4*) rats, and ACI control rats were used. All breeding was performed at the Animal Research Center at Erasmus MC, Rotterdam, The Netherlands. Animals were housed in individually ventilated cages under specific pathogen free (SPF) conditions as previously described [23, 26]. The protocol received approval from the Animal Ethical Committee of Erasmus MC.

Congenic rat strains were generated using a speed congenic strategy as previously described for the *Rf-1B* strain by Provoost et al [22]. A schematic view of the introgressed *Rf* regions of the various congenic strains is presented in Figure 1. The congenics generated here contain the earlier reported 95% CI for *Rf-1* and *Rf-4*. In the *Rf-1A* single congenic strain, the congenic region is on chromosome 1 between markers *D1Rat74* (227.2 Mb) and *D1Rat90* (267.3 Mb), spanning 40.1 Mb, about 15% of the chromosome. The *Rf-1A* region is about 17 Mb larger than the region in the *Rf-1B* strain [22].

In the *Rf-4* single congenic strain, the region is on chromosome 14 between markers *D14Mit11* (5.0 Mb) and *D14Rat82* (30.3 Mb), spanning 25.3 Mb, about 23% of the chromosome. In the *Rf-1A+4* double congenic rat the *Rf-1* congenic region is between *D1Mit18* (224.6 Mb) and *D1Rat90* (267.3 Mb), spanning 42.7 Mb. In the *Rf-1A+4* double congenic rat the *Rf-4* region is between the markers *D14Mit11* (5.0 Mb) and *D14Rat33* (29.3 Mb), spanning 24.3 Mb (i.e., 1.0 Mb smaller than in the *Rf-4* single congenic). In addition, a second region of chromosome 14 from FHH has been introgressed between markers *D14Rat65* (36.4 Mb) and *D14Rat90* (74.0 Mb), spanning 37.6 Mb. This region, however, is way outside the 95% CI for the *Rf-4* QTL (between 5 and 20 Mb) on chromosome 14. A whole genome scan with 140 to 150 genetic markers on these three congenic strains showed that there was no detected FHH genomic contamination on other chromosomes.

Renal damage susceptibility

Experiments for assessment of renal damage susceptibility were performed on 164 animals starting from the age of 6 to 7 weeks. Per strain, the animals were randomly divided over four treatment groups (Table 1). The first received no treatment, remaining with two kidneys (i.e., control situation). The second remained with two kidneys and was chronically treated with L-NAME (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) (two kidneys + L-NAME) to induce systemic hypertension. The third treatment consisted of renal mass reduction by unilateral nephrectomy, and the fourth of unilateral nephrectomy + L-NAME–induced hypertension.

Surgery for unilateral nephrectomy and L-NAME treatment were performed as previously described [27]. There was some variation in the amount of L-NAME intake. However, Figure 3 shows that blood pressure levels were equivalent among the different strains of rats within the different treatment groups. Urine of individual rats was collected after 6, 12, and 18 weeks of treatment. The animals were housed in metabolic cages (Tecniplast, Buggugiate, Italy). Urine was collected during 2 consecutive days after a 3-day adaptation period. Following the

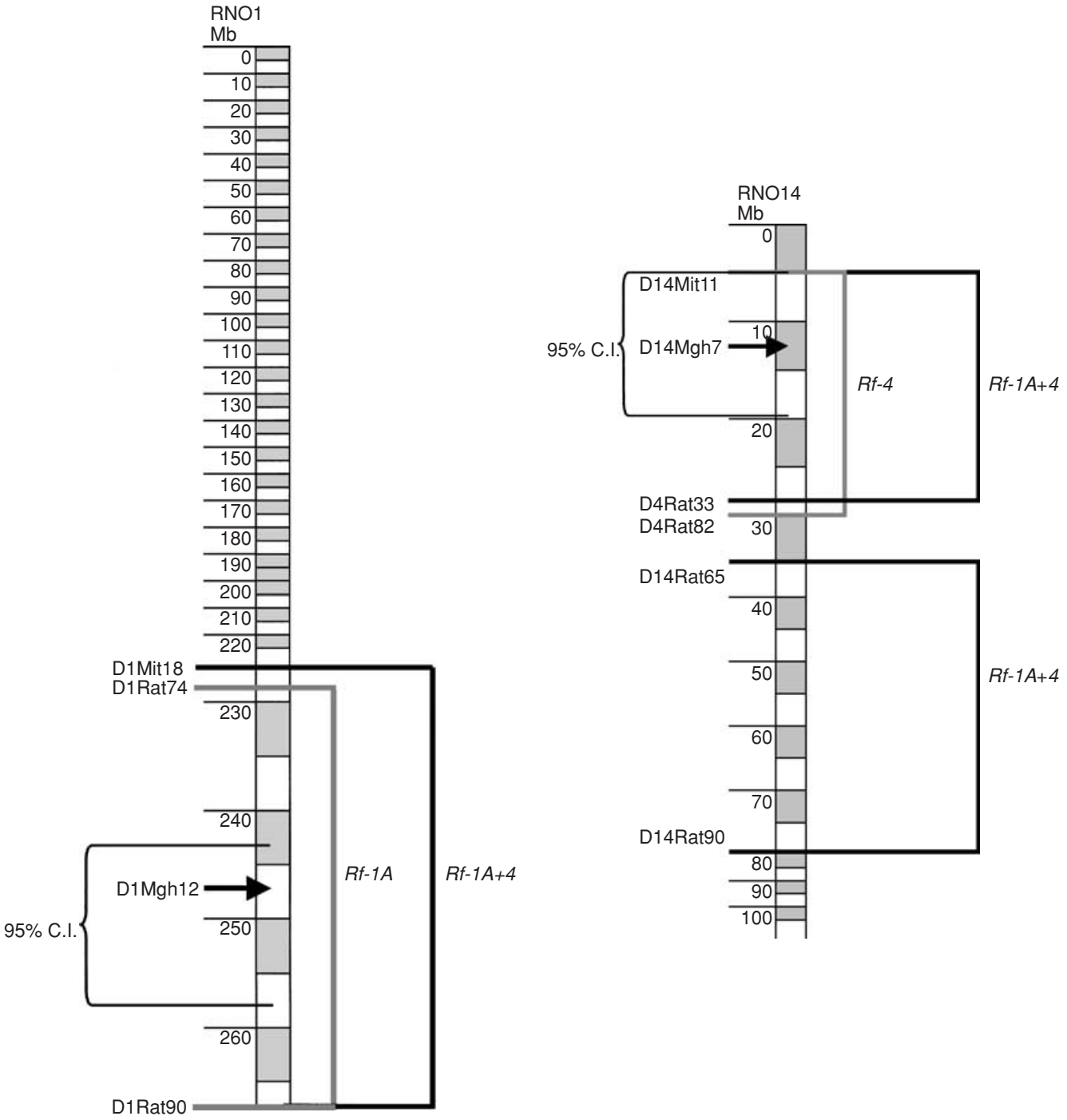

Fig. 1. Genetic maps of rat chromosomes 1 and 14 depicting the homozygous Fawn-Hooded hypertensive (FHH) regions introgressed on the August × Copenhagen Irish (ACI) background in the *Rf-1A*, *Rf-4*, and *Rf-1A+4* congenic strains. The areas homozygous FHH in the congenic strains are indicated by the solid and striped lines. The arrows indicate the locations of the *Rf-1* and *Rf-4* QTL peaks found in previous studies (i.e., *D1Mgh12* for *Rf-1* and *D14Mgh7* for *Rf-4* [34, 47]). Distances are given in megabase pairs (Mb). 95% CI represents the 95% confidence interval of the quantitative trait loci (QTLs).

urine collection, systolic blood pressure was measured by the tail-cuff method, using a photoelectric oscillatory detection device (IITC Life Science, Woodland Hills, CA, USA) in awake, restrained, but trained rats, as described previously [22].

Shortly after the last series of urine collections and systolic blood pressure measurements the animals were sacrificed as previously described [23]. Kidneys were collected and weighed. The left kidney was used for histologic examination. The extent of glomerular damage was

Table 1. Number of rats studied in the renal susceptibility experiments

	Two-kidney	Two kidney + L-NAME	Unilateral nephrectomy	Unilateral nephrectomy + L-NAME
August × Copenhagen Irish	6	6	10	9
Rf-1A	11	12	12	12
Rf-4	9	9	10	10
Rf-1A+4	12	10	12	14, 14, 10[a]

L-NAME is L-arginine analogue N-nitro-L-arginine methyl ester.
[a]Number of rats at first, second, and third follow-up, respectively.

determined in 1 μm sections stained with periodic acid-Schiff (PAS) reagent. In each animal, 50 glomeruli of the left kidney were examined for the presence of sclerotic lesions (i.e., segmental glomerular scarring, obliteration of glomerular capillaries, mesangial matrix expansion, and adhesion formation between tuft and Bowman's capsule). The extent of glomerular damage was expressed as the percentage of the glomeruli (% FSGS) showed one or more of these features [23].

RBF autoregulation

Experiments for assessment of renal blood flow autoregulation were performed on 54 animals (15 ACI, 12 *Rf-1A*, 15 *Rf-4*, and 12 *Rf-1A+4* rats) with an age of 13 to 15 weeks. To get an indication of the presence of renal damage, UPV and UAV were assessed using a 24-hour sample obtained before the autoregulation experiments, while at the end of the evaluation both kidneys were collected and weighed and the left kidney was used to determine the %FSGS, as previously described [23].

Animals were anaesthetized with a mixture of 3% Isoflurane®, 30% N_2O, and 60% O_2 and surgically prepared for autoregulation studies [18, 19]. After surgery and a 10-minute equilibration period, the relationship between the left kidney RBF and the renal perfusion pressure (RPP) was determined. The RBF was recorded as the RPP was lowered from 150 to 80 mm Hg in 10 mm Hg steps by tightening a clamp around the aorta, followed by a 3-minute equilibration period. To normalize the outcome of the individual rats, the RBF at a RPP of 100 mm Hg (RBF_{100}) was considered to be 100%. Renal autoregulatory indexes (RAIs) over the range of pressures from 80 to 150 mm Hg were calculated by the method of Semple and de Wardener [28]. A RAI of 0 indicates perfect autoregulation of RBF, and a RAI of 1 indicates that there is no autoregulation present due to a fixed renal vascular resistance.

Analytic procedures

Plasma and urinary samples were analyzed with an ELAN System (Eppendorf-Merck, Hamburg, Germany) using colorimetric assays. Total urinary protein was determined colorimetrically with pyrogallol red-molybdate complex [29]. Plasma and urinary albumin levels were determined with bromocresol green [30]. Plasma and urinary creatinine levels were determined with the Jaffé method without deproteinization [31].

Statistics

Data are presented as mean ± SEM, unless stated otherwise. Statistical differences in mean values between groups were compared using one-way analysis of variance (ANOVA), followed by the Bonferroni test to determine which pairs were significantly different. These tests were performed using the Primer of Biostatistics for Windows program (version 4.0) (McGraw Hill, 1996).

For different parameters (X) the magnitude of the effects of the presence of the *Rf-1* or *Rf-4* QTL on the ACI background and their interaction was calculated as follows:

Effect of *Rf-1* equals $X_{Rf-1A} - X_{ACI}$, effect of *Rf-4* QTL equals $X_{Rf-4} - X_{ACI}$, and the interaction between the *Rf-1* and *Rf-4* QTLs equals $X_{ACI} + X_{Rf1+4} - X_{Rf-1} - X_{Rf-4}$. In these formulas X_{strain} is the mean value of the parameter under investigation at the different time points for the various strains and treatments. The standard deviation (SD) was calculated as the weighed SD of the variables in the formulas.

Statistical significance of the main effect of the *Rf-1* and *Rf-4* QTLs and their interaction was calculated using a 2 × 2 factorial ANOVA procedure provided by VassarStats (*faculty.vassar.edu/lowry/anova2 × 2.html*). In all tests, a P value <0.05 was considered to be statistically significant.

RESULTS

Animal survival and albuminuria

All two-kidney, two-kidney + L-NAME, and unilateral nephrectomy rats survived the 18-week follow-up period. Following unilateral nephrectomy + L-NAME treatment, four of the 14 *Rf-1A+4* double congenic rats did not survive up to the third measurement. Data obtained from these four rats are included in the results for the first and second measurements.

Both UPV and UAV values were assessed, and no remarkable differences were found between the two values. Mean values for UAV during follow-up from the various treatments are presented in Figure 2. In the two-kidney situation, UAV was significantly higher in *Rf-1A+4*

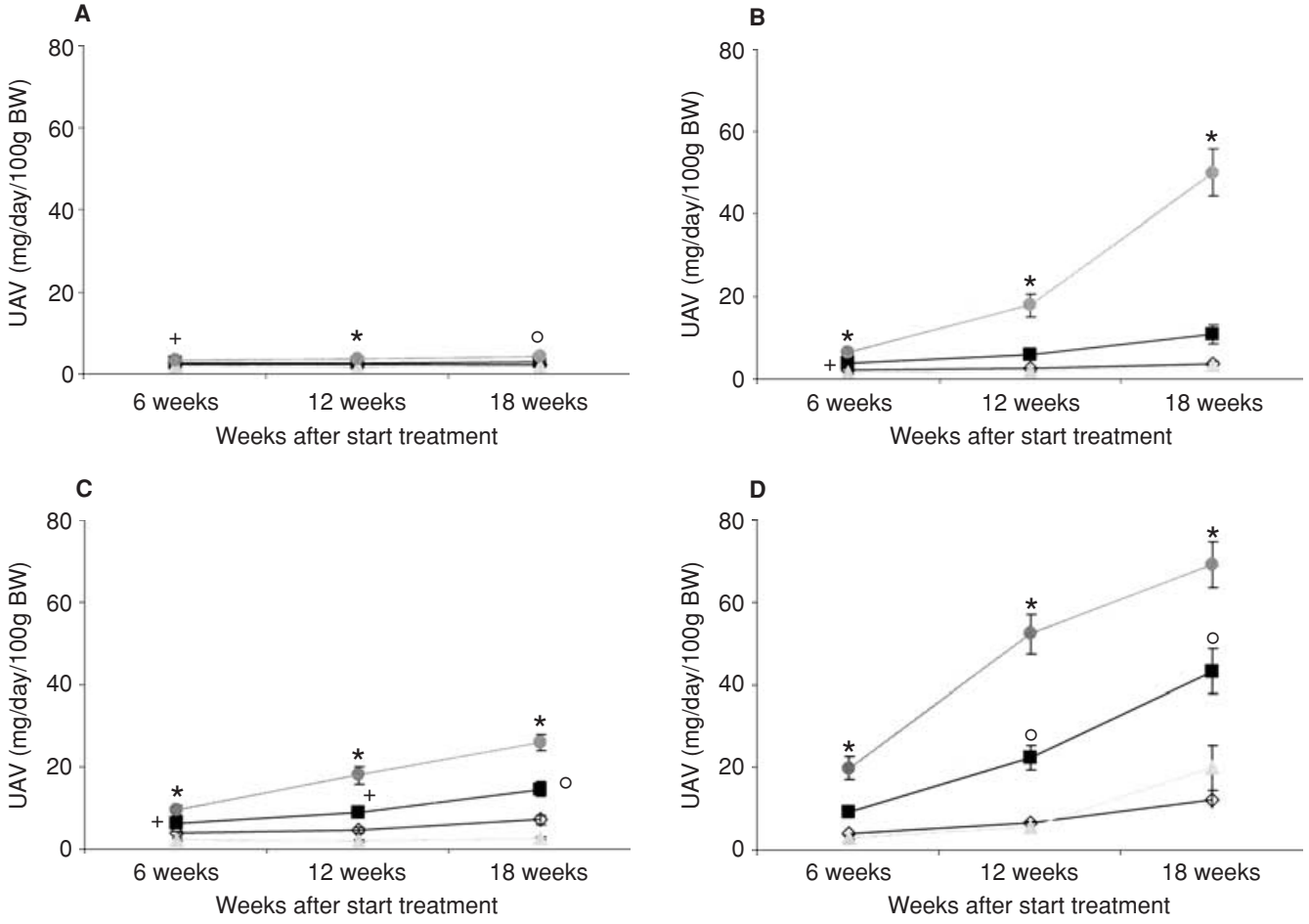

Fig. 2. Albuminuria (UAV) after 6, 12, and 18 weeks of follow-up during four treatments on August × Copenhagen Irish (ACI) (◇), *Rf-1A* (■), *Rf-4* (▲), and *Rf-1A+4* (●) rats. (*A*) Two-kidney. (*B*) Two-kidney + L-arginine analogue N-nitro-L-arginine methyl ester (L-NAME)–induced hypertension. (*C*) Unilateral nephrectomy. (*D*) Unilateral nephrectomy + L-NAME–induced hypertension. Values (mg/day per 100 g body weight) are given as mean ± SEM, number of rats is given in Table 1. $^{*}P < 0.05$ vs. ACI, *Rf-1A*, and *Rf-4*; $^{\circ}P < 0.05$ vs. ACI and *Rf-4*; $^{+}P < 0.05$ vs. *Rf-4*.

double congenic rats compared to *Rf-4* single congenics at all time points, to ACI at the second and third time point, and to *Rf-1A* single congenic rats at the second time point (Fig. 2A). Following two-kidney + L-NAME treatment, UAV in *Rf-1A+4* double congenic rats was significantly increased at all time points compared to ACI, *Rf-4* and *Rf-1A* single congenic rats (Fig. 2B). Following unilateral nephrectomy, UAV was at all time points significantly higher in *Rf-1A+4* double congenics compared to ACI, or *Rf-4* and *Rf-1A* single congenic rats. The level of UAV in *Rf-1A* single congenic rats was significantly higher compared to *Rf-4* single congenics at all time points and to ACI rats at the third time point (Fig. 2C). When treated with unilateral nephrectomy + L-NAME, UAV in *Rf-1A+4* double congenic rats at all time points was significantly increased compared to ACI, *Rf-4* and *Rf-1A* single congenic rats. In *Rf-1A* single congenics rats this treatment led, at the second and third evaluation, to a significantly increased UAV compared to ACI and *Rf-4* single congenic rats (Fig. 2D). Regardless of treatment or time point, no statistically significant differences in UAV were found in the *Rf-4* single congenic rats when compared to ACI (Fig. 2).

Systolic blood pressure

Values for systolic blood pressure are presented in Figure 3. In the two-kidney situation, *Rf-1A* single congenic rats compared to ACI showed a small, but significant increase in systolic blood pressure at the first and second evaluations (Fig. 3A). After unilateral nephrectomy, a slightly higher systolic blood pressure was present in the *Rf-4* single congenics compared to ACI at the second time point (Fig. 3C). Chronic L-NAME treatment increased systolic blood pressure in all strains. At the second and third evaluations, stylolic blood pressure in *Rf-1A+4* double congenic rats with two-kidney + L-NAME treatment was significantly increased compared to ACI and *Rf-1A* single congenic rats. At the first time point, following two-kidney + L-NAME treatment, *Rf-4* single

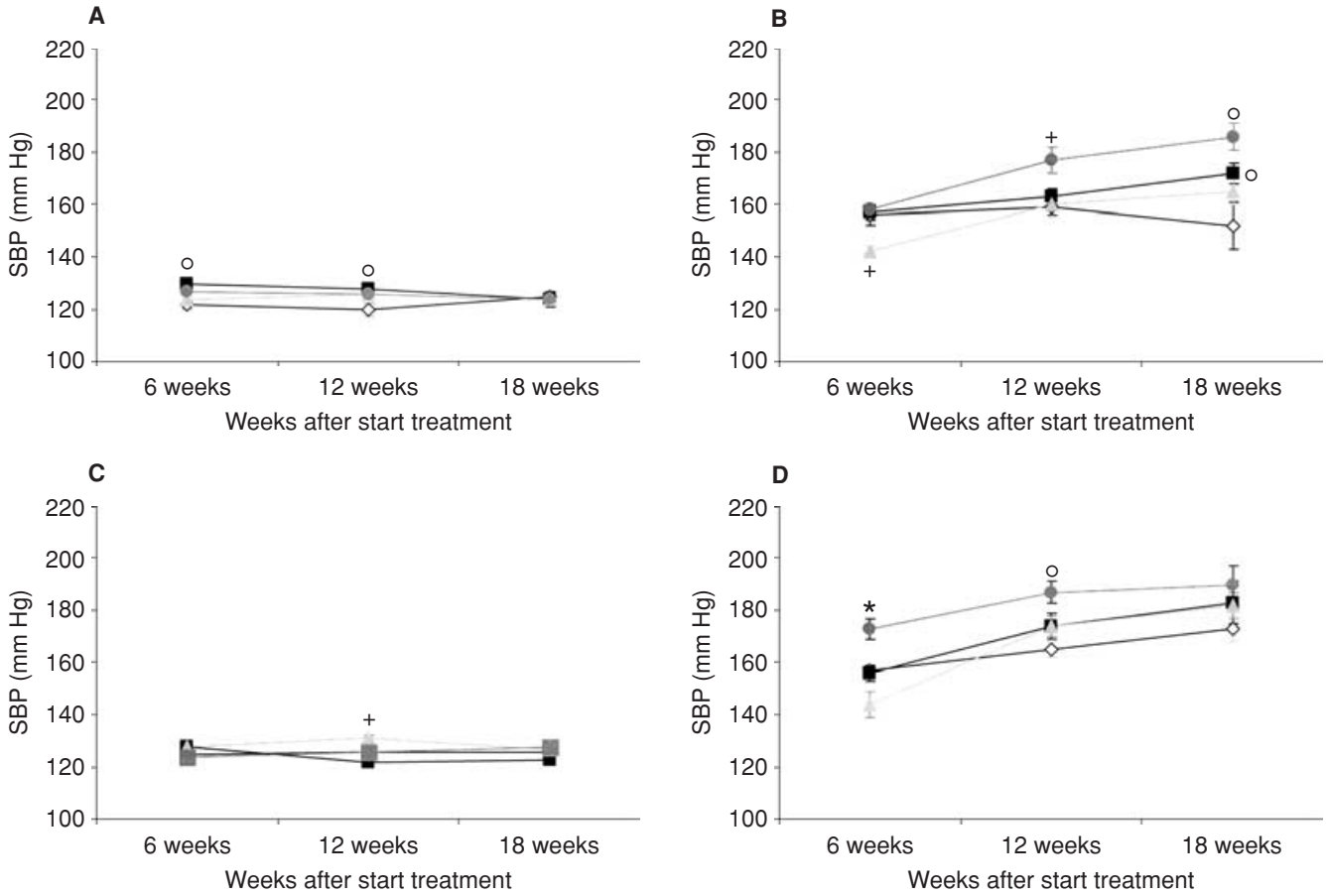

Fig. 3. Tail-cuff blood pressure at 6, 12, and 18 weeks in August × Copenhagen Irish (ACI) (◇), *Rf-1A* (■), *Rf-4* (▲), and *Rf-1A+4* (●) rats. (*A*) Two-kidney. (*B*) Two-kidney + L-arginine analogue N-nitro-L-arginine methyl ester (L-NAME)–induced hypertension. (*C*) Unilateral nephrectomy. (*D*) Unilateral nephrectomy + L-NAME–induced hypertension. Values (mm Hg) are given as mean ± SEM; number of rats is given in Table 1. $^{*}P < 0.05$ vs. ACI, *Rf-1A*, and *Rf-4*; $^{+}P < 0.05$ vs. ACI and *Rf-1A*; $^{\circ}P < 0.05$ vs. ACI. SBP is systolic blood pressure.

congenic rats had a significantly lower systolic blood pressure compared to ACI and *Rf-1A*. This difference was not seen at the second and third evaluations (Fig. 3B). The *Rf-1A+4* double congenics with unilateral nephrectomy + L-NAME treatment, showed a higher systolic blood pressure at the first and second evaluations compared to ACI and to *Rf-1A* single congenics at the second evaluation. However, at the final evaluation there was no difference present (Fig. 3D).

Gene-gene and gene-treatment interactions

The results of the 2 × 2 factorial ANOVA analyses showed the presence of significant gene-gene interactions. While *Rf-4* alone showed no effect on UAV when compared to ACI, combining *Rf-4* with *Rf-1A* always resulted in a further increase in UAV when compared with *Rf-1A* single congenics. Furthermore, the magnitude of these interactions was significant in all four treatments. However, the greater the hemodynamic stress upon the kidney, the greater the synergistic effect.

An example is presented in Figure 4, for the UAV at the second time point, after 12 weeks of treatment. It is shown that changing the *Rf-1* genotype from homozygous ACI (AA) to homozygous FHH (FF), while the *Rf-4* genotype remains AA, induced an increase in UAV per 100 g body weight in all treatment groups. In contrast, changing the *Rf-4* genotype from AA to FF, while the *Rf-1* genotype remains AA, showed almost no change in UAV. Assuming an additive effect on UAV when both *Rf-1* and *Rf-4* change from AA to FF, an expected change in UAV can be calculated. In all four situations, the observed changes in UAV significantly exceeded the expected change. The difference, being the interactive effect between *Rf-1* and *Rf-4* depended on the experimental situation, but was significant in all treatment groups. The magnitude was small with two-kidney (+1.5 mg/day/100 g body weight), intermediate with two-kidney + L-NAME (+12.5 mg/day/100 g body weight), and unilateral nephrectomy (+11.7 mg/day/100 g body weight) and large in the unilateral nephrectomy + L-NAME situation (30.9 mg/day/100 g body weight).

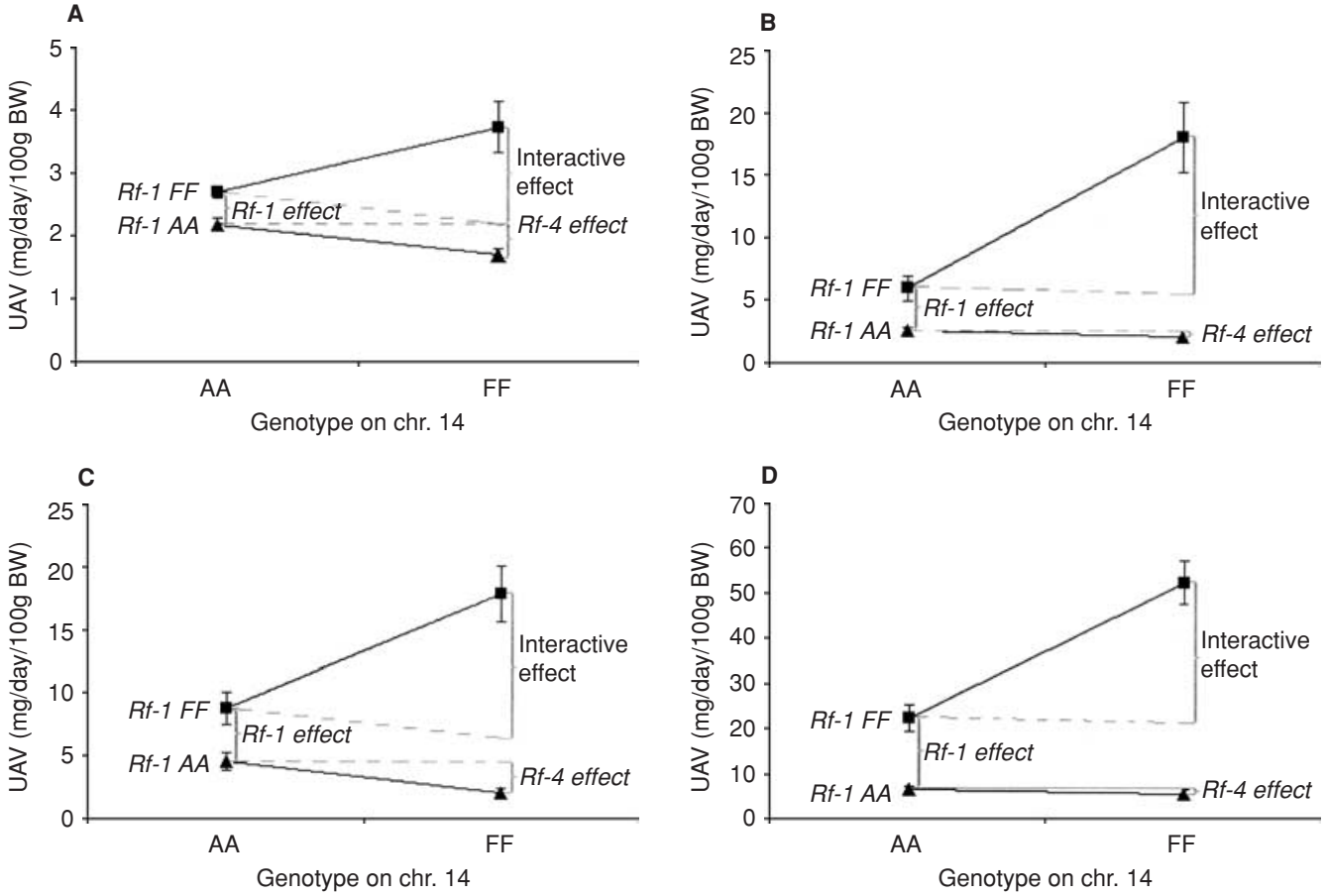

Fig. 4. The effects of the *Rf-1* and *Rf-4* (chromosome 14) genotype and their interaction of *Rf-1* and *Rf-4* on albuminuria (UAV) after 12 weeks of treatment. (*A*)Two-kidney. (*B*) Two-kidney + L-arginine analogue N-nitro-L-arginine methyl ester (L-NAME)–induced hypertension. (*C*) Unilateral nephrectomy. (*D*) Unilateral nephrectomy + L-NAME–induced hypertension. Abbreviations are: AA, homozygous August × Copenhagen Irish (ACI); FF, homozygous Fawn-Hooded hypertensive (FHH). Values for UAV (mg/day per 100 g body weight) are mean ± SEM. Statistics are given in Table 2.

Additional information about the effects of the *Rf-1* and *Rf-4* QTLs and their interactions on UAV at all three time points is provided in Table 2. The major finding being that the magnitude of the interaction between *Rf-1* and *Rf-4* not only depended on the type of treatment but also on the duration of the treatments (i.e., the longer the treatment the larger the interaction).

Findings at end of experiment

The incidence of focal segmental glomerulosclerosis (%FSGS), creatinine clearance per 100 g body weight, and plasma albumin level are summarized in Table 3. The%FSGS was significantly higher in *Rf-1A+4* double congenic rats compared to ACI, *Rf-4* and *Rf-1A* single congenic rats, regardless of treatment. Following both two-kidney + L-NAME and unilateral nephrectomy + L-NAME, the %FSGS was significantly higher in *Rf-1A* single congenics compared to ACI rats. Irrespective of the treatment, no statistically significant differences in %FSGS were found between *Rf-4* single congenics and ACI rats.

As with UAV, a significant interactive effect upon the%FSGS was present between the *Rf-1* and *Rf-4* QTLs. The magnitude of the interaction differed per treatment. It was low (+9 ± 6%) ($P = 0.021$) for two-kidney, intermediate ((+21 ± 10%) $P = 0.002$) for two-kidney + L-NAME and for unilateral nephrectomy (+24 ± 10%) ($P < 0.001$), and most pronounced for unilateral nephrectomy + L-NAME treatment (+49 ± 12%) ($P < 0.001$).

In the two-kidney and unilateral nephrectomysituation, creatinine clearance per 100 g body weight level was significantly lower in *Rf-1A* single congenic and *Rf-1A+4* double congenic rats compared to *Rf-4* single congenics. Following two-kidney + L-NAME, creatinine clearance per 100 g body weight was lower in *Rf-1A+4* double congenics compared to all other strains, while in *Rf-1A* single congenic rats it was lower compared to *Rf*-4 single congenic rats. After unilateral nephrectomy + L-NAME treatment, creatinine clearance per 100 g body weight of *Rf-1A+4* double congenics and *Rf-1A* single congenics

Table 2. Effect of the *Rf-1* and *Rf-4* quantitative trait loci (QTLs) and their interaction on albuminuria (UAV)

	6 weeks of treatment	2 × 2 factorial ANOVA *P* value	12 weeks of treatment	2 × 2 factorial ANOVA *P* value	18 weeks of treatment	2 × 2 factorial ANOVA *P* value
Two-kidney						
Rf-4 effect	−0.3 ± 0.9	$P = 0.476$	−0.5 ± 0.4	$P = 0.234$	−0.3 ± 0.6	$P = 0.178$
Rf-1 effect	+0.6 ± 0.5	$P < 0.001$	+0.5 ± 0.4	$P < 0.001$	+0.9 ± 0.7	$P < 0.001$
Interaction of *Rf-1* and *Rf-4*	+0.8 ± 0.8	$P = 0.072$	+1.5 ± 0.8	$P = 0.009$	+1.6 ± 1.3	$P = 0.045$
Two-kidney + L-NAME						
Rf-4 effect	−0.8 ± 1.0	$P = 0.116$	−0.5 ± 0.9	$P = 0.002$	−0.3 ± 2.1	$P < 0.001$
Rf-1 effect	+1.6 ± 1.3	$P < 0.001$	+3.4 ± 2.9	$P < 0.001$	+7.1 ± 6.9	$P < 0.001$
Interaction of *Rf-1* and *Rf-4*	+3.3 ± 1.4	$P < 0.001$	+12.5 ± 5.1	$P < 0.001$	+39.5 ± 10.7	$P < 0.001$
Unilateral nephrectomy						
Rf-4 effect	−1.5 ± 1.4	$P = 0.155$	−2.5 ± 1.7	$P = 0.010$	−4.5 ± 2.8	$P = 0.011$
Rf-1 effect	+2.2 ± 2.0	$P < 0.001$	+4.2 ± 3.6	$P < 0.001$	+7.4 ± 5.2	$P < 0.001$
Interaction of *Rf-1* and *Rf-4*	+4.8 ± 2.6	$P = 0.004$	+11.7 ± 4.7	$P < 0.001$	+15.9 ± 5.2	$P < 0.001$
Unilateral nephrectomy + L-NAME						
Rf-4 effect	−1.0 ± 1.4	$P = 0.003$	−0.9 ± 3.3	$P < 0.001$	+7.7 ± 13.0	$P < 0.001$
Rf-1 effect	+5.1 ± 2.7	$P < 0.001$	+15.7 ± 6.9	$P < 0.001$	+30.9 ± 14.7	$P = 0.012$
Interaction of *Rf-1* and *Rf-4*	+11.8 ± 6.4	$P = 0.005$	+30.9 ± 11.3	$P < 0.001$	+18.2 ± 16.1	$P = 0.009$

Abbreviations are: ANOVA, analysis of variance; L-NAME, L-arginine analogue N-nitro-L-arginine methyl ester; ACI, August × Copenhagen Irish. For each treatment and time point the *Rf-4* effect ($UAV_{Rf-4} - UAV_{ACI}$), the *Rf-1* effect ($UAV_{Rf-1A} - UAV_{ACI}$), and the interaction ($UAV_{Rf-1A+4} - UAV_{Rf-1A} - UAV_{Rf-4} + UAV_{ACI}$) were calculated. Data represent change in UAV (mg/day per 100 g body weight) ± SD. Statistical significance of the overall (main) effect of *Rf-1* and *Rf-4* and their interaction were calculated using 2 × 2 factorial ANOVA.

Table 3. Measurements at end of follow-up

	Number	Body weight *g*	FSGS *% glomeruli*	Creatinine clearance *mL/min/100 g body weight*	Plasma albumin *g/L*
Two-kidney					
August × Copenhagen Irish	6	303 ± 4	10 ± 2	0.55 ± 0.05	28.8 ± 0.9
Rf-4	9	317 ± 7	8 ± 1	0.66 ± 0.05	28.9 ± 0.6
Rf-1A	11	326 ± 5	15 ± 2[c]	0.45 ± 0.03[b]	28.9 ± 0.5
Rf-1A+4	12	327 ± 5[a]	22 ± 2[d]	0.44 ± 0.03[b]	28.6 ± 0.5
ANOVA		$P = 0.037$	$P < 0.001$	$P < 0.001$	$P = 0.977$
Two-kidney + L-NAME					
August × Copenhagen Irish	6	312 ± 8	14 ± 4	0.54 ± 0.02	29.1 ± 0.2
Rf-4	9	309 ± 4	6 ± 1	0.62 ± 0.03	29.3 ± 0.3
Rf-1A	12	326 ± 4	26 ± 2[c]	0.47 ± 0.02[b]	27.7 ± 0.5
Rf-1A+4	10	278 ± 6[d]	39 ± 5[d]	0.34 ± 0.03[d]	25.8 ± 0.8[d]
ANOVA		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Unilateral nephrectomy					
August × Copenhagen Irish	10	309 ± 6	18 ± 2	0.50 ± 0.03	27.8 ± 0.4
Rf-4	10	298 ± 4	6 ± 2	0.56 ± 0.03	28.2 ± 0.3
Rf-1A	12	324 ± 6[c]	26 ± 3[c]	0.46 ± 0.02[b]	26.3 ± 0.5
Rf-1A+4	12	331 ± 5[c]	38 ± 5[d]	0.45 ± 0.02[b]	25.0 ± 0.4[d]
ANOVA		$P < 0.001$	$P < 0.001$	$P = 0.014$	$P < 0.001$
Unilateral nephrectomy + L-NAME					
August × Copenhagen Irish	9	301 ± 10	20 ± 3	0.52 ± 0.03	27.2 ± 0.6
Rf-4	10	283 ± 6	16 ± 6	0.51 ± 0.02	27.2 ± 0.8
Rf-1A	12	320 ± 5[c]	42 ± 3[c]	0.37 ± 0.02[c]	23.2 ± 1.0[c]
Rf-1A+4	14	258 ± 10[c]	69 ± 3[d]	0.34 ± 0.04[c]	23.5 ± 0.7[c]
ANOVA		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Abbreviations are FSGS, focal segmental glomerulosclerosis, ANOVA, analysis of variance; L-NAME, L-arginine analogue N-nitro-L-arginine methyl ester. Values are given as mean ± SEM.

[a] $P < 0.05$ vs. August × Copenhagen Irish; [b] $P < 0.05$ vs. *Rf-4*; [c] $P < 0.05$ vs. August × Copenhagen Irish and *Rf-4*; [d] $P < 0.05$ vs. August × Copenhagen Irish, *Rf-4*, and *Rf-1A*.

was lower than that of *Rf-4* single congenics and ACI rats. At the end of the follow-up, plasma albumin level was significantly decreased in *Rf-1A+4* double congenic rats compared to all other strains after two-kidney + L-NAME or unilateral nephrectomy treatment, and compared to ACI and *Rf-4* single congenics after unilateral nephrectomy + L-NAME treatment. Following unilateral nephrectomy + L-NAME treatment, *Rf-1A* single congenics also showed a lower plasma albumin level in comparison to ACI and *Rf-4* single congenic rats.

Assessment of RBF autoregulation

No differences were found in the absolute values of the RBF at 100 mm Hg between ACI, *Rf-1A*, *Rf-4*, and *Rf-1A+4* rats. Despite the relatively large variations within

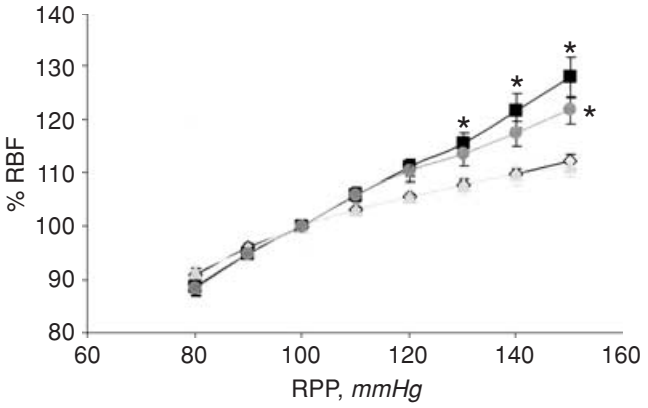

Fig. 5. Renal blood flow (RBF) autoregulation curves in two-kidney August × Copenhagen Irish (ACI) (◇) ($N = 15$), *Rf-1A* (■) ($N = 12$), *Rf-4* (▲) ($N = 15$), and *Rf-1A+4* (●) ($N = 12$) rats. Values [as %RBF at renal perfusion pressure (RPP) of 100 mm Hg] are given as means ± SEM (error bars). $^*P < 0.05$ vs. ACI and *Rf-4*.

each strain, a significantly higher RBF at a RPP of 150 mm Hg was present in the *Rf-1A* and *Rf-1A+4* rats in comparison with ACI rats (data not shown). After normalizing the RBF, more significant differences were revealed (Fig. 5). From 130 to 150 mm Hg, mean %RBF values were significantly higher in the *Rf-1A* single congenics compared to ACI rats. At 150 mm Hg, mean %RBF value of *Rf-1A+4* double congenics was significantly higher compared to ACI rats. This points to an impairment of the RBF autoregulation in *Rf-1A* and *Rf-1A+4* rats. Over the pressure range 100 to 150 mm Hg, the RAIs were in the order of 0.2 to 0.3 in ACI as well as in *Rf-4* single congenics, indicating a normal renal autoregulation in these strains. In contrast, RAI values were significantly increased to levels of about 0.4 to 0.7 in *Rf-1A* single and *Rf-1A+4* double congenic rats, indicating an impaired renal autoregulation.

No marked renal damage was present in the rats used for the autoregulation experiment. In all four strains, the average UAV level was about 2 to 4 mg/day/100 g body weight, while the percentage of injured glomeruli was in the order of 2% to 6%.

DISCUSSION

The major finding of our present study is the presence of a powerful synergistic interaction between the *Rf-1* and *Rf-4* QTLs that is markedly enhancing the susceptibility to renal damage as measured by the levels of UAV during and the %FSGS at the end of the follow-up. The magnitude of the interactive effect differs between the various treatments, and appears to depend on the intensity and the duration of the exposure to the various stressors. The largest effect is observed when a reduction in renal mass by unilateral nephrectomy is combined with L-NAME–induced hypertension. A moderate effect is noted following unilateral nephrectomy or L-NAME–induced hypertension. Even in the two-kidney situation there appears to be a small synergistic interaction.

The interpretation of the present finding of an interaction between the *Rf-1* and *Rf-4* QTL is complicated by the presence of a second part of the FHH chromosome 14 introgressed in the *Rf-1A+4* double congenic rats. However, this extra part located between 36.4 and 74.0 Mb is way off the 95% CI of the *Rf-4* QTL (i.e., between 5 and 20 Mb). In theory, a gene or genes in this additional part could account for the increased susceptibility of the double congenic strain. Preliminary data, obtained by Jacob and Lutz (Lutz et al, manuscript in preparation), exclude a role of the distal part of chromosome 14. In order to narrow down the interval of the *Rf-4* QTL, a panel of subcongenic strains was generated from the *Rf-1A+4* and *Rf-1A* strains tested in the present experiments. One of the subcongenic strains (*Rf1A+4_by*) was identical to the *Rf-1A+4* with regard to the distal part of chromosome 14, but the FHH genotype between the markers *D14Mit11* (4.9 Mb) and *D14Mit2* (19.1 Mb) was replaced by ACI. In these strains susceptibility to renal damage was compared with *Rf-1A* single congenics. Following 8 weeks of unilateral nephrectomy and L-NAME treatment UAV corrected for body weight in the *Rf-1A+4_by* subcongenic (34 ± 6 mg/day) ($N = 6$) (mean ± SEM) was significantly less that that of *Rf-1A+4* double congenics (98 ± 10 mg/day) ($N = 20$), but not different from that of *Rf-1A* single congenics (31 ± 4 mg/day) ($N = 6$). These findings indicate that the gene(s) responsible for the interaction between the *Rf-1* and *Rf-4* QTLs is located on chromosome 14 in the 4.9 and 19.1 Mb interval. Future studies aim to further reduce the size of the *Rf-4* interval and positionally clone the *Rf-4* gene(s).

Nature of the interaction

In recent years, there is an increasing awareness that genetic and environmental factors play an important role in common complex diseases in humans, such as diabetes, hypertension, and mental disorders [32–34]. Double congenic rat strains have been studied to establish interactions between blood pressure QTLs [24, 25]. To our knowledge, the present findings are the first to directly show a QTL interaction increasing susceptibility to renal damage. Such an interaction between the *Rf-1* and *Rf-4* QTLs was predicted in an F2 population from a cross of FHH and ACI rats subjected to unilateral nephrectomy [21]. The synergistic interaction is extremely powerful in this protective genomic background, particularly in the presence of kidney stressors. This observation could be important in understanding the variability found in the human situation.

Gene-gene interactions may have different phenotypic implications [35]. For example, the interaction could be

additive, where the combined effect of *Rf-1* and *Rf-4* in the double congenic strain would roughly be equal to the sum of the effects of *Rf-1* and *Rf-4* on the phenotype. Irrespective of treatment the effect of *Rf-4* on UPV and UAV was only marginal, and therefore additivity can be excluded for UPV and UAV, but not for FSGS. The UPV and UAV results clearly show that the gene-gene interaction has a powerful synergistic effect, consistent with epistasis for complex traits [36]. Our findings fit this concept. The effects of changing the genotype of *Rf-1* and *Rf-4* from homozygous ACI to homozygous FHH has a powerful and significant effect on the pathophysiologic parameters of renal damage. In addition, the complexity of the gene-gene interaction is further distended by the fact that the effects on renal damage of changing the *Rf-1* and *Rf-4* genotypes also depends on the severity of the (hemodynamic) strain put upon the kidney and the length of the exposure to the harmful stimuli. The renal damage parameters (UAV and FGS) are distant traits (i.e., not likely to be directly related to a gene defect). With the generation of the *Rf-1A+4* double congenics we have constructed a relatively simple two-loci model that results in an increased susceptibility to renal damage. One effect of *Rf-1* appears to be an impairment of the renal autoregulation, which might result in an increased P_{GC} when faced with systemic hypertension and/or reduced renal mass. The effect of *Rf-4* appears to be on a different level and only becomes detectable presence two copies of the *Rf-1* QTL from FHH. We can only speculate about possible mechanism(s) of action of *Rf-4*. It is conceivable that *Rf-4* as well as other *Rf*-QTLs play a role in protecting the integrity of the glomerular filtration barrier when exposed to an increased P_{GC}. Although this limits the number of possible candidates, it still leaves a wide range of molecular pathways important in the biology of the podocyte and the glomerular barrier [37–40].

Next to five QTLs in FHH, numerous QTLs influencing parameters of renal damage have been detected in other strains [41–46]. The Rat Genome Database (*http://rgd.mcw.edu*) has collected over 90 QTLs associated with renal damage or renal function components, some of them overlapping with the five *Rf*-QTLs. As far as renal damage is concerned, it looks as if 15 to 20 different loci might be involved in the various rat models [1]. Should a similar number of QTLs also play a role in humans, the number of possible interacting gene combinations that can be derived from these loci becomes tremendous. Inbred rat models remain relevant for gene identification and gene interaction, as the number of gene combinations per strain is relatively small. With the detection of an interaction between *Rf-1* and *Rf-4*, we continue to investigate the interaction between *Rf-1* and the three other *Rf*-QTLs. The ultimate goal, of course, is to identify the genes in the various *Rf*-QTLs, as recently accomplished for *Rf-2* [47], and establish how they affect susceptibility to renal damage and how they interact [48]. Subsequently, these genes and gene-gene interactions can be tested in humans. With the recent completion of the sequencing of the rat genome [49] it has become feasible to make a rat, mouse, and human genome comparison [50]. Analysis of the genes present in the various *Rf*-regions with the Ensembl Genome Browser (*http://www.ensembl.org*) indicated that the 25 Mb *Rf-1* interval on rat chromosome 1 contains about 200 known and predicted genes with homologies in both mouse and human. The 25 Mb *Rf-4* interval on rat chromosome 14 contains about 130 known and predicted genes. Currently, 15% to 25% of the genes are predicted, novel, genes without a description of the gene product, while the description of others is vague. Eventually, shortening the congenic region will further decrease the number of candidate genes.

CONCLUSION

The present studies show that there is an interaction between the *Rf-1* and *Rf-4* QTL, which is markedly enhancing the susceptibility to renal damage in ACI genomic background that is ordinarily protective for proteinuria. It appears that the *Rf-1* QTL of the FHH rat contains one or more genes directly influencing renal susceptibility, possibly by impairing the efficacy of renal autoregulation. The *Rf-4* QTL also contains one or more genes that influence renal susceptibility. However, the effect of the *Rf-4* QTL can only be observed in the homozygous presence of the *Rf-1* QTL from the FHH rat.

ACKNOWLEDGMENTS

Studies were performed with financial support from grants from the Medical and Health Research NWO program (902-18-299); and National Institutes of Health (NIH-R01-HL69321). The last one is a subcontract from a grant provided to Dr. H.J. Jacob at the Medical College of Wisconsin. The authors like to thank Mr. P. Van Schalkwijk (Erasmus MC) for his excellent technical assistance.

Reprint requests to Sabine J. van Dijk, B.Sc., Department of Paediatric Surgery, Room WK 211, Erasmus MC, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands.
E-mail: a.provoost@erasmusmc.nl

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